BIOSYNTHESIS OF NATURAL PRODUCTS—AN OVERVIEW OF CURRENT RESULTS AND PROBLEMS. II. STUDIES OF NITROGEN-CONTAINING METABOLITES.[1]

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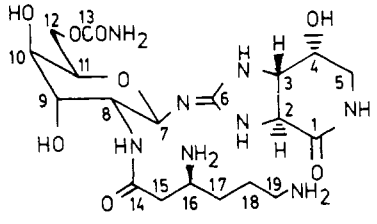
State-of-the-art nmr techniques available for observing ^{13}C, ^{2}H, ^{17}O, ^{31}P and ^{15}N nuclei have greatly simplified the study of biosynthetic and metabolic pathways and, today, can provide answers to questions that could not have been reasonably considered even a few years ago. For example, when two stable isotopes are included at strategic positions in the same precursor molecule, the intramolecularity of biochemical reactions can be determined. This double-label approach also provides a ready window for observing bond-making, bond-breaking, and bond-conserving processes. The utility of these techniques is demonstrated by the following studies of a variety of nitrogen-containing metabolites.

Streptothricin F (**1**), a complex metabolite representative of a large family of broad-spectrum antibiotics (1), contains eight nitrogen atoms of diverse, although



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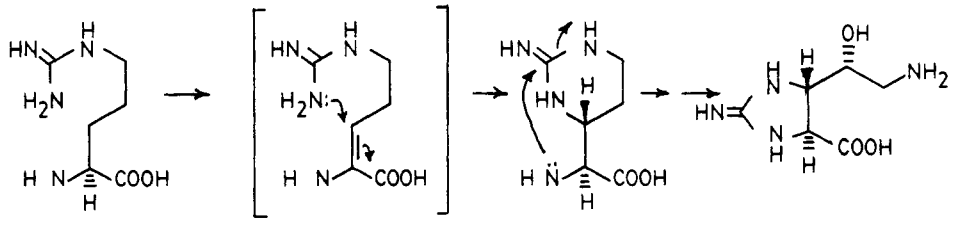
potentially identifiable, origins. A rational biogenesis of the heterocyclic portion (streptolidine (**2**)) from arginine (**3**), originally proposed by Bycroft and King in 1972, (2) is presented in scheme 1. When we began our work, the β-lysine portion of **1** was expected to be derived from α-lysine (3–5), although the mechanism of the transformation remained to be determined.



3 **2**

After the necessary preliminary ^{14}C feedings, we fed sodium [1,2-^{13}C] acetate to *Streptomyces* L–1689–23. (6) By pulsing the acetate both at the beginning of the fermentation and at the beginning of antibiotic production, we hoped to get a general view of the primary metabolism providing the key precursors to the anti-

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biotic. At the same time, by using the doubly-labeled acetate, we hoped to clarify seemingly conflicting claims in the literature for the role of acetate specifically in the biosynthesis of **2**. The ^{13}C nmr spectrum of **1** having been previously assigned, the complete labeling pattern of **1a** was easily determined. As shown in table 1, nine singlets of the broad band decoupled spectrum were flanked by doublets due to the ^{13}C-^{13}C spin-coupling of acetate units incorporated intact, and two other singlets showed substantial ^{13}C enrichment due to incorporation of (eventually) cleaved acetate units.

TABLE 1. Incorporation of sodium [1,2-$^{13}C_2$] acetate into streptothricin F.[a]

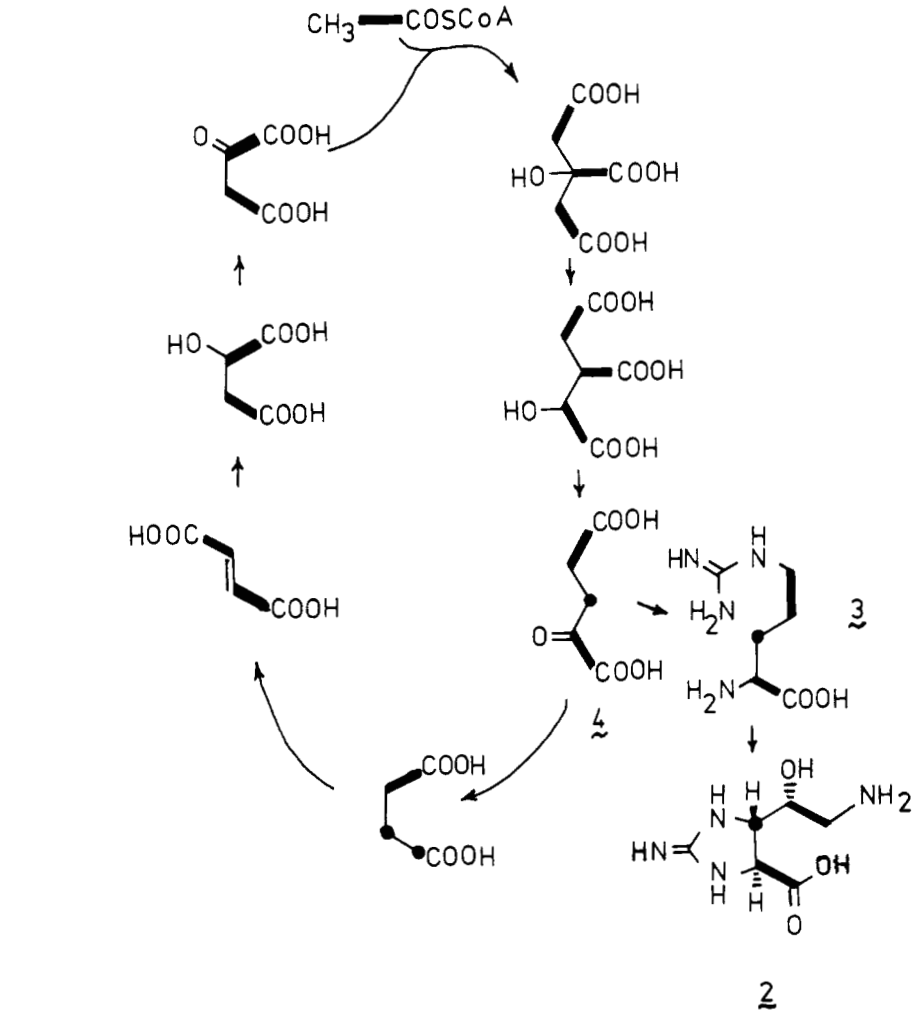
Assignment	Chemical Shift, δ[b]	J_{CC}, Hz	% Enhancement[c]	% Enrichment[d] (±0.2)
1	167.7	55.5	—[e]	0.2
2	52.1	55.5	0.6	0.5
3	58.9		0.9	
4	58.5	37.0	0.1	2.3
5	46.8	37.0	0.1	1.9
6	160.4		—[e]	
7	76.5		—	
8	46.6		0.1	
9	71.1		0.0	
10	67.6		0.0	
11	64.1		0.1	
12	57.9		0.0	
13	155.4		—[e]	
14	169.7	48.1	—[e]	0.1
15	34.2	48.1	0.4	0.3
16	45.9	37.0	0.5	0.2
17	26.9	37.0, 35.1	0.5	0.5
18	20.5	35.1	0.2	0.2
19	36.6		0.8	

[a]Spectra taken in D_2O containing 2% pyridine.
[b]Based on the middle pyridine signal = 135.5 ppm.
[c]Excess ^{13}C over natural abundance in the central peaks of each carbon resonance, normalized relative to the C-7 signal = 1.1% ^{13}C.
[d]^{13}C enrichment of the satellites, normalized relative to the C-7 signal = 1.1% ^{13}C.
[e]Not measured.

Pairing the doublets by their coupling constants showed that the labeling pattern of the streptolidine portion was, as expected, predictable if acetate were metabolized to arginine via α-ketoglutarate (**4**) derived from the Kreb's Cycle, with the arginine then metabolized as proposed in scheme 1. Thus, as shown in scheme 2, acetate served as a specific, but indirect, precursor of **2**.

The remaining ^{13}C labels were all in the β-lysine portion of the antibiotic. Examination of two spectra of **1** obtained consecutively revealed that the C-17 satellites were actually two overlapping doublets with nearly identical coupling constants. The presence of two populations of labeled antibiotic, one with an intact acetate unit at C-16/C-17 and the other with an intact acetate unit at C-17/C-18, revealed the involvement of a symmetrical biosynthetic intermediate. In fact, of the two pathways in microorganisms known to lead to α-lysine (**5**), only the diaminopimelic acid pathway could provide the observed labeling (scheme 3). Epimerization at either chiral center of S,S-diaminopimelic acid (**6**) would normally provide the same *meso* form (**7**), but in this case the unsymmetrical ^{13}C labeling destroyed this degeneracy. The data clearly indicated that β-lysine (**8**) was derived by rearrangement of α-lysine.

With these strong indications of arginine and α-lysine as the direct primary precursors, we next embarked on a simultaneous investigation of the carbon and nitrogen metabolism of these two amino acids. We did this using ^{13}C nmr spectroscopy and precursors doubly labeled with ^{13}C and ^{15}N. (7,8) Each experiment was


CH3COSCoA
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HO
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HOOC
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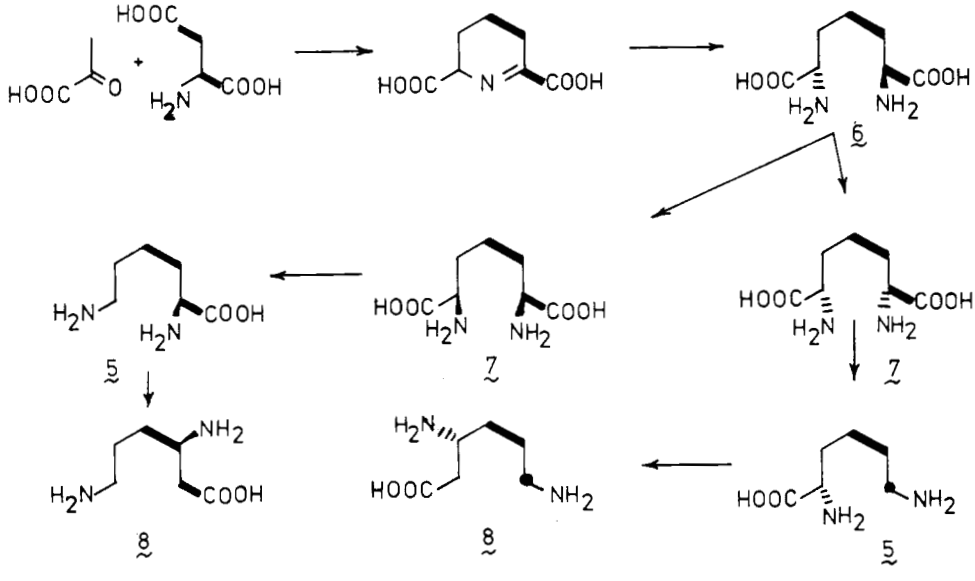
HOOC
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5
HOOC
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NH2
7
HOOC
COOH
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NH2
7
H2N
NH2
HOOC
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H2N
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COOH
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NH2
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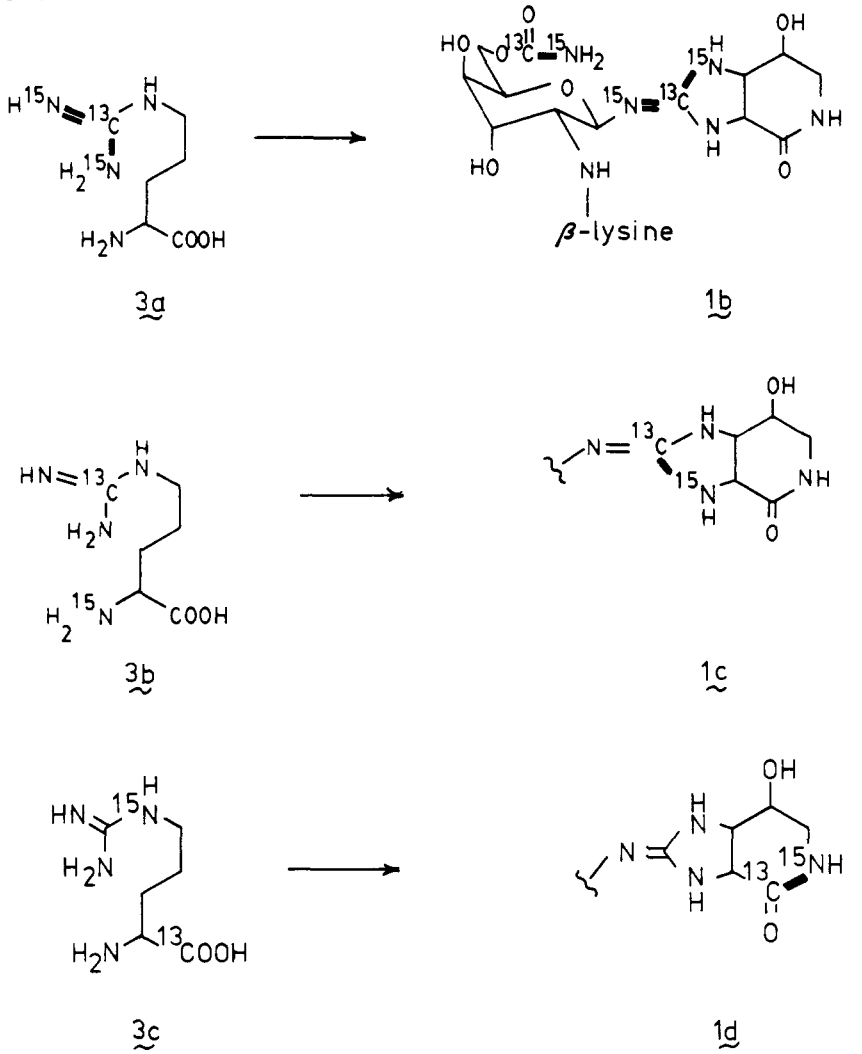
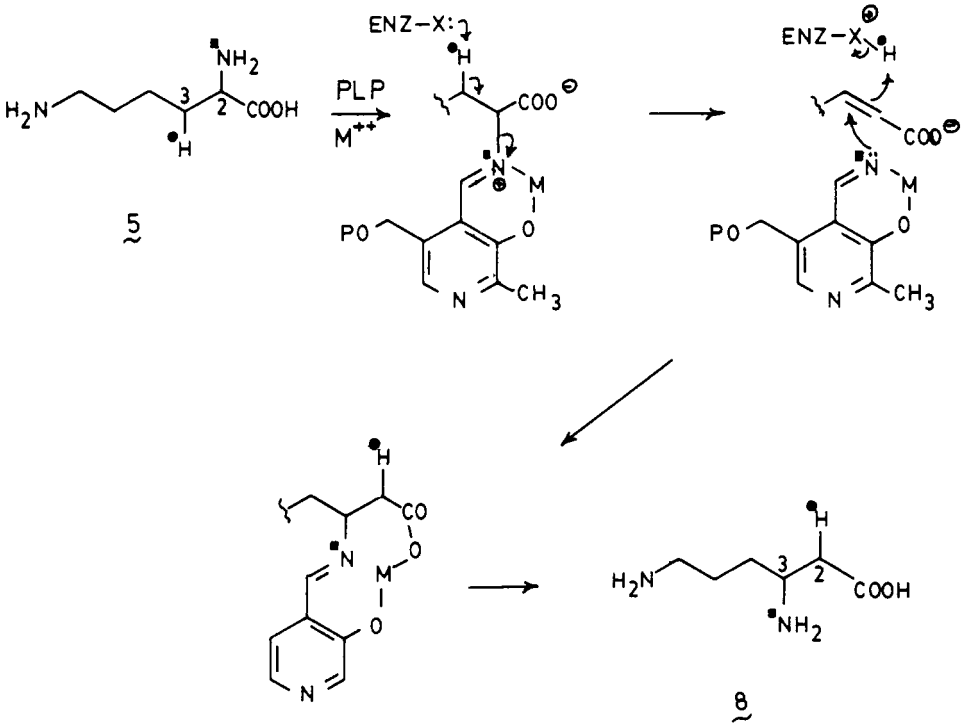
designed so that the enriching ^{15}N in the antibiotic would be detected through its spin-coupling to a directly bonded ^{13}C label.

Initially, three arginines, **3a-3c**, (9) were prepared and incorporated, yielding labeled streptothricins **1b-1d**. From the observed new signals in the ^{13}C nmr spectra, metabolic bond-conserving (**1b**) and bond-making (**1c** and **1d**) processes were detected. As anticipated, the proposal in scheme 1 was consistent with the metabolism revealed in these experiments, and the heteronuclear spin-coupling technique was able to easily solve what would have otherwise been a formidable problem.



In addition to being produced biosynthetically by aerobic *Streptomyces*, β-lysine is also the first product in the catabolism of **5** by anaerobic *Clostridia*. (10) From preliminary data reported on the *Clostridium* reaction using a cell-free preparation, (11), we believed that a double migration was involved: nitrogen migrated from C-2 to C-3 and hydrogen migrated from C-3 to C-2, with pyridoxal phosphate involved, possibly as shown in scheme 4. In the hope that a similar process was operating in *Streptomyces*, [3-^{13}C, 2-^{15}N] lysine **5a** was synthesized and incorporated. (12) As shown in scheme 5, a large new doublet for C-16 in the ^{13}C nmr spectrum of the antibiotic proved that the original α-amino group had, indeed, been retained and that it had migrated to C-3 by an intramolecular process.



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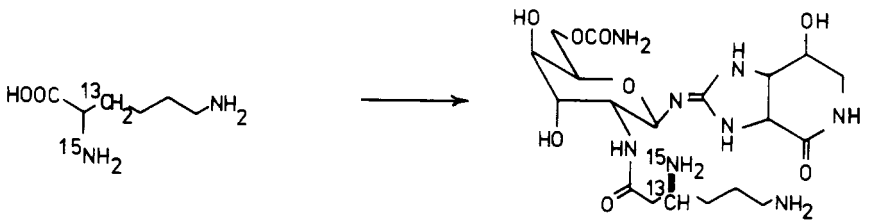
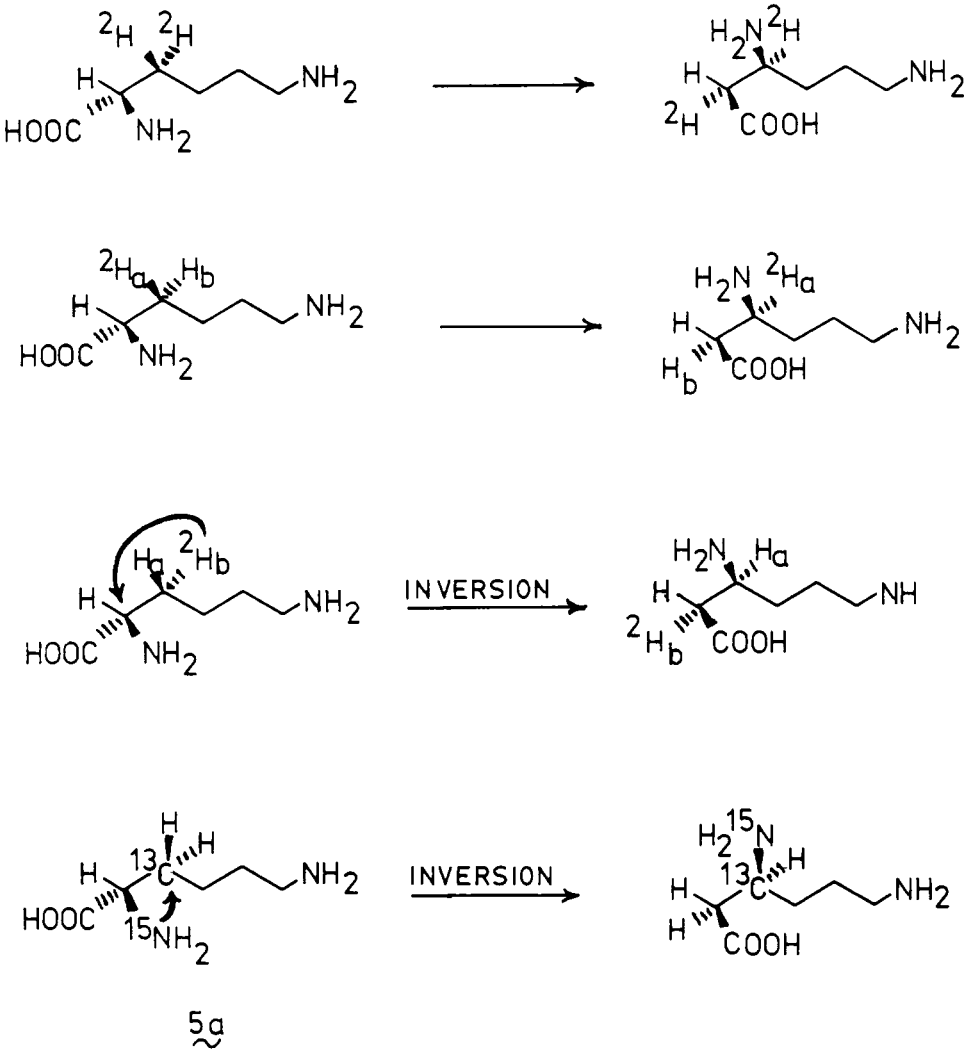
While we were examining β-lysine biosynthesis in this *Streptomyces*, Dr. John Aberhart of the Worcester Foundation for Experimental Biology had been studying the *Clostridium* enzyme reaction using deuterated lysines and ^2H nmr for analysis. We are now collaborating in order to complete studies of both organisms. As shown in scheme 6, it has been found that the *Clostridium* enzyme causes migration of the 3-proR hydrogen of **5** to C-2 with inversion of stereochemistry, (13) and that, consistent with the *Streptomyces* result, nitrogen undergoes an intramolecular migration from C-2 to C-3, also with inversion. (14) These results reveal yet a fourth distinctly different mechanism for 2,3- amino acid mutase reactions (15), a surprising situation with possible evolutionary implications. The deuterated lysines are now being fed to *Streptomyces* L-1689-23.



5a

We have also examined a different lysine metabolism, that of the L-lysine decarboxylase reaction of *Bacillus cadaveris*, using a different nmr technique. This reaction had previously been studied by Spenser using tritium labels, and a chemical degradation of the derived cadaverine to glycine which was then analyzed enzymatically (16). In our approach, a deuterated cadaverine **9a** was produced by the enzymatic decarboxylation of **5** in D_2O, and the epimer **9b** was produced by decarboxylation of α-deuterated lysine **5b** in H_2O. Unlabeled cadaverine and each of the deuterated cadaverines were converted to their biscamphanamides **10a-10c**, and their ^1H nmr spectra taken in the presence of Eu(fod)$_3$.



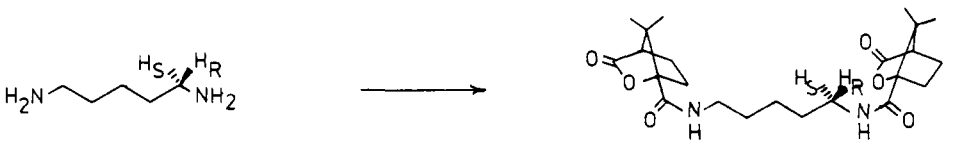
5a

5

9a

5b

9b



9: $H_R = H_S = H$

9a: $H_R = {}^2H$

b: $H_S = {}^2H$

10a: $H_R = H_S = H$

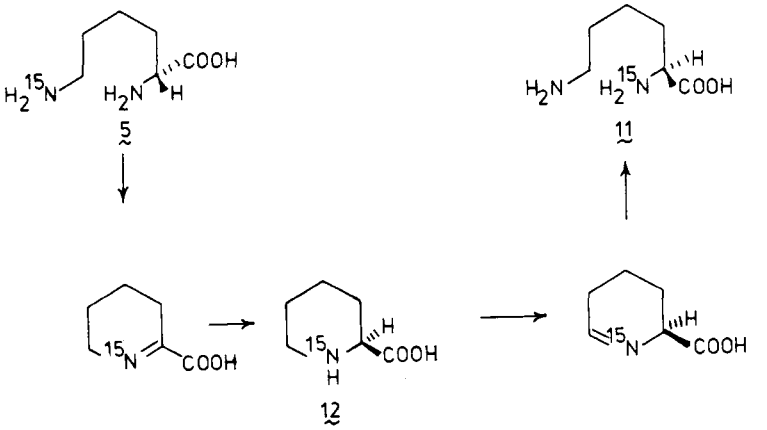
b: $H_R = {}^2H$

c: $H_S = {}^2H$

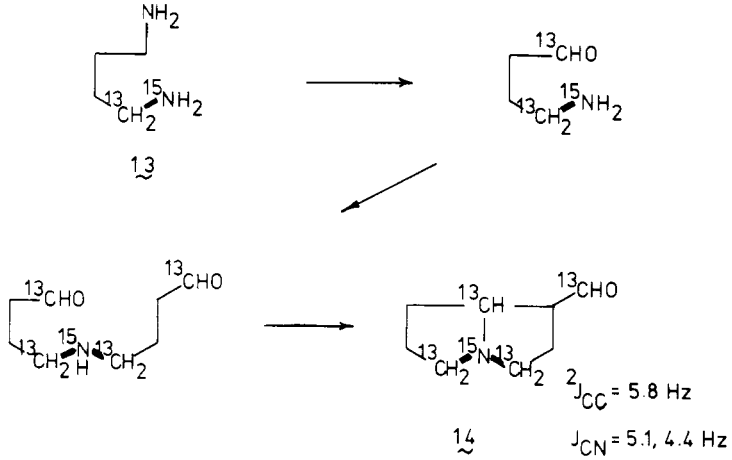
As first shown by Gerlach and Zagalak (17) for camphanate esters and later shown for glycine (18) and GABA (19) camphanamides, the technique can distinguish between the prochiral hydrogens of a methylene adjacent to an ester oxygen or an amide nitrogen. These hydrogens, initially enantiotopic, become diastereotopic in the camphanyl derivatives. Due to the geometry of the complex of these derivatives with the Europium shift reagent, the H_S hydrogen is always shifted to a lower field than the H_R hydrogen. With 9.2 mole % of shift reagent, the proR and proS hydrogens of **10a** were cleanly separated, and each signal integrated for the same area (two hydrogens each due to the symmetry of the molecule). As expected on the basis of Spenser's results, under identical spectral conditions the H_R signal of **10b** integrated for half of the H_S signal, and this result was reversed in the spectrum of **10c**. Thus, retention of configuration occurred, and the new hydrogen occupied the same geometrical position as had the carboxyl group.

The ease of this approach is striking, and we are currently using it to study bacterial L-ornithine decarboxylase (ODC) and L-arginine decarboxylase. Using tritium labels and tritium nmr, we plan to use the same approach to study the mammalian ODC enzyme (from rat prostate) involved in spermidine biosynthesis; this enzyme cannot be obtained in sufficient quantity to use deuterium.

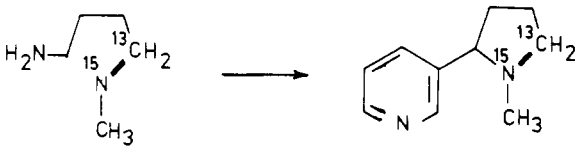
Completing this overview of lysine metabolism, Leistner has used ^{15}N nmr spectroscopy to study the conversion of L-lysine to D-lysine (**11**) by *Neurospora crassa*. (20) Initially, mass spectrometry revealed that [2-^{15}N]-L-lysine lost the ^{15}N label in this conversion, while the ^{15}N label of [6-^{15}N]-L-lysine was retained in **11**. The ^{15}N nmr spectrum of **11** produced in the latter experiment revealed that the label was now at C–2. Since L-pipecolic acid **12** had previously been implicated, Leistner has suggested the pathway shown in scheme 7.



A number of studies recently reported by other groups have also used the ^{13}C-^{15}N heteronuclear spin-coupling technique. Spenser reported the use of doubly-labeled putrescine **13** to show that a symmetrical intermediate is involved in



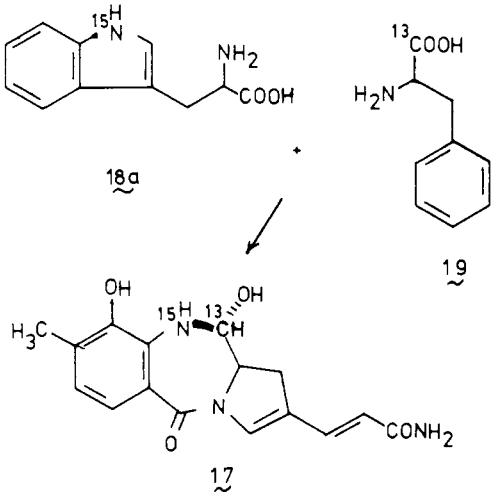
retronecine (**14**) biosynthesis (21). Leete has used doubly-labeled *N*-methylputrescine (**15**) to show that a symmetrical intermediate is not involved in its conversion to nicotine (**16**) (22). These two studies used higher plants. Hurley

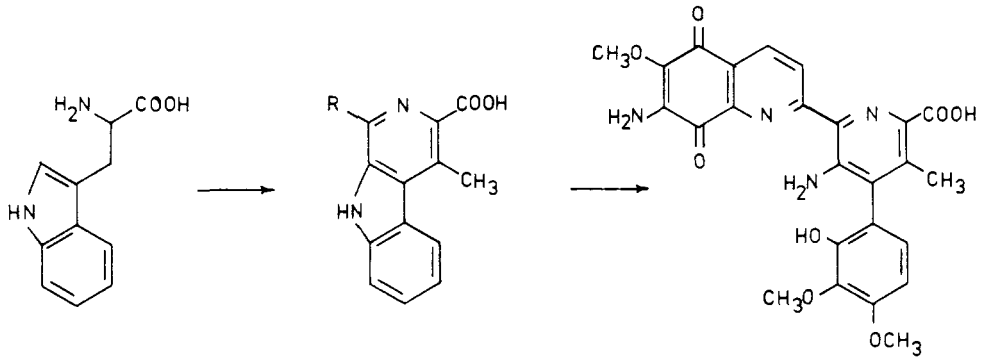


15 16

has shown, using anthramycin (**17**) biosynthesis, that high enrichments of different labeled precursors (tryptophan **18a** and phenylalanine **19**) can lead to observable spin-coupling with the covalent joining of the two biosynthetic units (23).

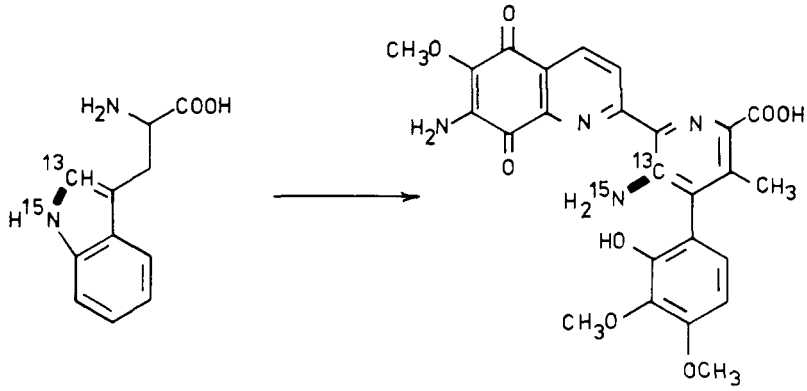
We have been studying the biosynthesis of streptonigrin (**20**), a very potent anticancer antibiotic, for some years. Tryptophan (**18**) was shown to be the precursor of the phenylpicolinic acid portion, apparently via a β-carboline intermediate **21**, which we suggested underwent hydroxylation and ring cleavage, as





18 21 20

shown in Scheme 8 (24). In order to determine the site of ring cleavage, [2-^{13}C, $^{15}N_b$] tryptophan **18b** was synthesized and fed to *Streptomyces flocculus*. From the spin-coupled doublet that flanked the C–5 natural abundance singlet in the ^{13}C nmr spectrum of **20a**, it was clear that the original indole nitrogen had been retained and that an unprecedented bond cleavage had occurred (25).

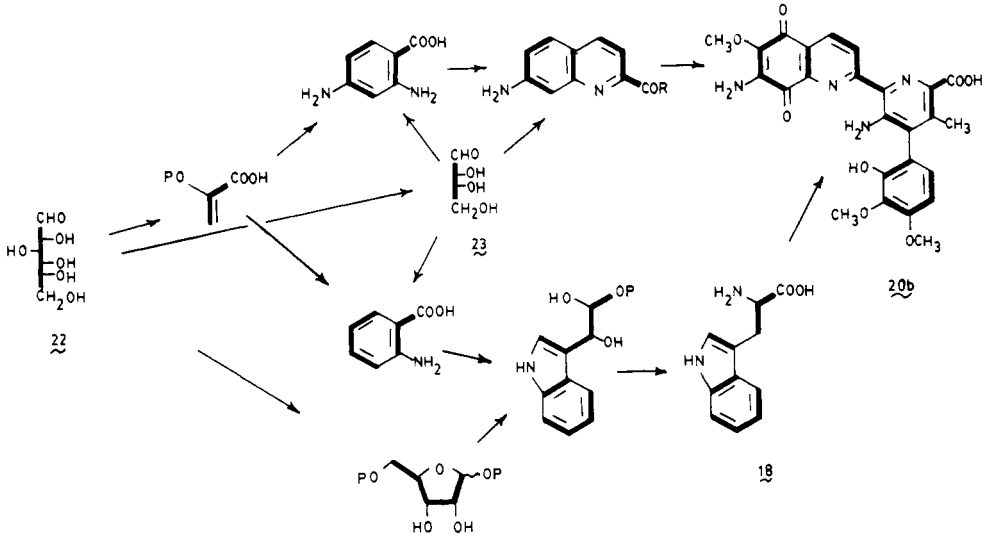


18b 20a

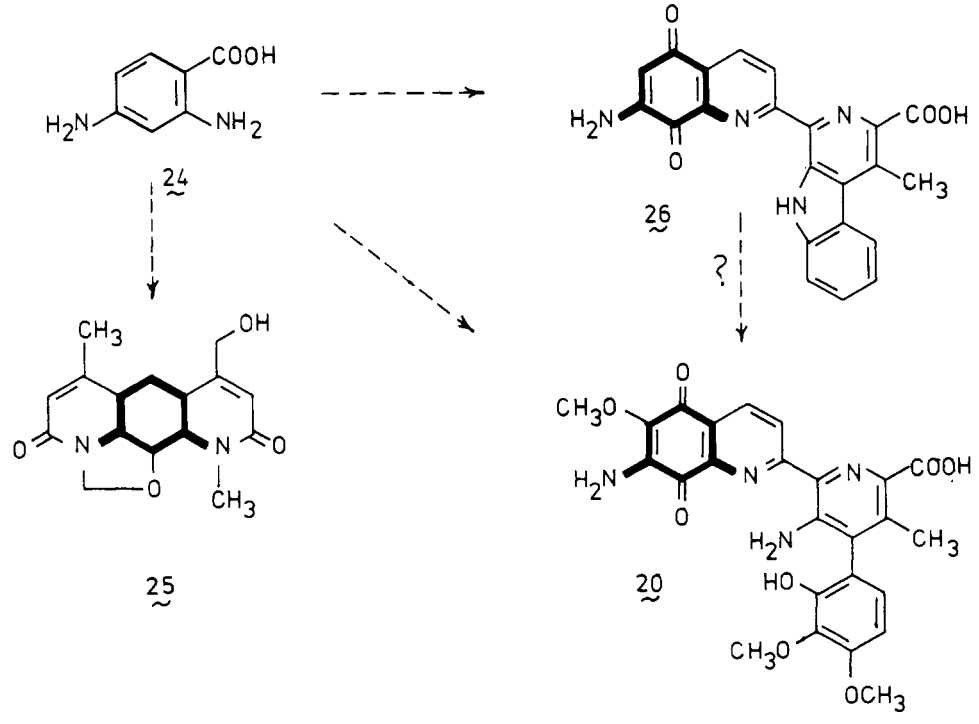
Results from a variety of feedings indicated that none of the known pathways producing quinoline rings was involved in streptonigrin biosynthesis. (25) In order to sort through all conceivable chemically-reasonable new biogenetic hypotheses, [UL-$^{13}C_6$]-D-glucose (**22**) was fed to *S. flocculus*, and its modes of incorporation into **20** were analyzed by ^{13}C nmr spectroscopy in collaboration with Professor David Cane of Brown University (26). The rationale for the experiment was that **22** would be metabolized to a large array of primary metabolites, each multiply-labeled with ^{13}C. Any ^{13}C-^{13}C bond retained through the various primary and secondary metabolic pathways would be observable from the homonuclear spin-couplings in the ^{13}C nmr spectrum of the antibiotic **20b**.

The spectrum of **20b** showed that each carbon had spin-couplings to at least one neighbor. At 67 MHz nearly all signals were clearly separated and first-order. From analysis of the multiplicity of each signal and a matching of coupling constants, it was possible to identify the size and location of most of the biogenetic units. With the biosynthesis of tryptophan and its mode of incorporation into **20** both already known, a convenient internal standard was available to simplify

the analysis. By selective ^{13}C-^{13}C spin-decoupling experiments, it was then possible to unequivocally confirm the initial analysis and reveal the one biogenetic unit (C-6', C-2, C-3, C-4) that had been obscured. Scheme 9 shows the labeling pattern of **20b**, with the biosynthesis of tryptophan and a reasonable pathway to the quinoline portion outlined. Experiments to confirm the utilization of three erythrose-4-phosphate (**23**) units in the biosynthesis of **20** are underway.

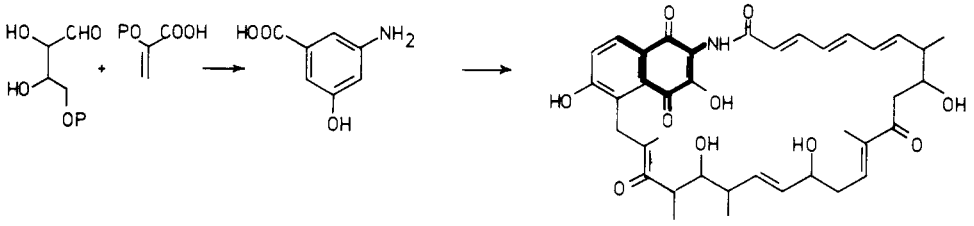


It appears likely now that 4-amino anthranilic acid (**24**) is an intermediate in the biosynthesis of **20**. We view **24** as representing a "C_6N_2" structural unit that reveals a significant biogenetic relationship with nybomycin (**25**), (27) as shown in scheme 10. The structure of a new antibiotic, lavendamycin (**26**), has recently been determined (28) and lends further support to this view, as well as to the suggested intermediacy of a β-carboline in the biosynthesis of **20**. Thus, the "C_6N_2"



unit appears to be a previously unrecognized, but significant, variation on the basic shikimate pathway leading to the common aromatic amino acids.

Finally, one of the most important recent biosynthetic findings has been the solution to the identity of the "C_7N" unit that has been recognized for nearly a decade as a structural unit of such diverse metabolites as the numerous ansamycins, the mitomycins and pactamycin. Two groups (29,30) have now shown that this unit, produced by another variation of the shikimate pathway, is 3-amino, 5-hydroxybenzoic acid (**27**), shown in scheme 11 for the biosynthesis of actamycin, **28**.



27 **28**

In presenting these examples, representing efforts in a dozen research groups, I have tried to show the elegant chemistry of Nature's approach to synthesizing complex molecules, as well as to show the cleverness of Man in devising ways to reveal and understand that chemistry.

ACKNOWLEDGEMENTS

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